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STAT1, NF- κ B and ERKs play a role in the induction of lipocalin-2 expression in adipocytes^{*}



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ABSTRACT

Lipocalin-2 (LCN2) is induced in conditions of obesity and Type 2 diabetes (T2DM). IFN γ and TNF α induce LCN2 expression in adipocytes in a manner that is dependent on transcription. The effects of these cytokines are additive. IFN γ induced STAT1 and TNF α induced NF- κ B play a role in the induction of LCN2. In the LCN2 promoter, one NF- κ B binding site and four STAT1 binding sites were identified by *in silico* and *in vitro* approaches. MAPK (ERKs 1 and 2) activation was required for the IFN γ and TNF α induction of LCN2 expression, but did not affect the nuclear translocation or DNA binding activity of STAT1 or NF- κ B. The NF- κ B binding site and the STAT1 binding sites we identified *in vitro* were confirmed by *in vivo* studies. Transfection of a LCN2 promoter/luciferase reporter construct confirmed acute activation by IFN γ and TNF α . Our studies identify mechanisms involved in the actions of cytokines secreted from immune cells in adipose tissue that induce LCN2 expression in conditions of obesity and T2DM.

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Keywords Lipocalin-2; STAT1; NF- κ B; ERKs; Adipocyte; TNF α ; IFN γ

1. INTRODUCTION

Adipose tissue is a major insulin sensitive tissue that plays key roles in regulating energy metabolism and insulin sensitivity [1–3]. Chronic inflammation and infiltration of immune cells in adipose tissues has been demonstrated to modulate adipocyte function and result in alterations of hormone secretion and insulin sensitivity [4,5]. TNF α and IFN γ are important pro-inflammatory cytokines that are known to be secreted from immune cells that infiltrate into adipose tissues [4,6,7]. These immune cells have the ability to induce insulin resistance in both cultured adipocytes and experimental animal models [6,8–10]. IFN γ signals via activating Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT1) signaling pathway and extracellular signal-regulated kinases (ERKs) mediated signaling pathway [10,11]. TNF α utilizes various signaling pathway and can stimulate both nuclear factor kappa B (NF- κ B) signaling pathway and ERKs mediated signaling pathway [12,13].

Lipocalins are a family of proteins that bind and transport small or hydrophobic molecules with conserved ligand binding sites [14]. As a member of the lipocalin family, lipocalin-2 (LCN2), also called neutrophil gelatinase-associated lipocalin (NGAL), was initially discovered as a matrix metalloproteinase 9 (MMP-9) binding protein that attenuated MMP-9 degradation [15]. Studies have shown that LCN2 increases during exposure to invading bacteria [16] and can be bound to bacterial siderophores to limit bacterial growth by reducing iron uptake [17,18]. More recent research has revealed that LCN2 is secreted from mature adipocytes [19]. Expression of LCN2 is significantly up-regulated in

conditions of insulin resistance and type 2 diabetes (T2DM) [20]. Human studies indicate that circulating LCN2 positively correlates with adiposity, triglyceride, blood glucose level, insulin resistance, and is negatively related to high-density lipoprotein cholesterol [21,22]. However, the roles of LCN2 in the pathology of adipose tissue insulin resistance are still unclear. There is evidence that LCN2 can induce the expression of PPAR γ and adiponectin [23] and that administration of LCN2 attenuates the inhibitory effect of TNF α on insulin-stimulated glucose uptake [23]. However, other data indicates that knockdown of LCN2 improves insulin action in adipocytes [20]. In order to clarify the functions of LCN2 in whole body insulin sensitivity, LCN2 knockout mice have been generated by several labs. However, the results of these studies have further clouded our understanding of the functions of LCN2 and the topic is even more controversial. In one study, global LCN2 deficiency caused dyslipidemia, fatty liver and insulin resistance [24]. On the contrary, a separate study indicated that LCN2 null mice had improved aging and obesity-mediated insulin resistance [25], supporting the notion that the lack of LCN2 is metabolically favorable. The most recent characterization of LCN2 deficient mice indicates that LCN2 deficiency did not have a significant effect on age or obesity-induced insulin resistance [26]. Collectively, these studies suggest that the roles of LCN2 in modulating insulin sensitivity are still unclear. Nonetheless, there are numerous studies and clear and convincing evidence to demonstrate that modulation of LCN2 in mice and humans significantly affects insulin sensitivity. Our studies have focused on understanding the regulation of LCN2 expression by factors that contribute to the pathogenesis of insulin resistance.

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To enhance our understanding of LCN2 in obesity and insulin resistance, we performed mechanistic studies to examine how LCN2 expression is induced in adipocytes. Our results demonstrate that the increase of LCN2 expression is mediated by pro-inflammatory cytokines that are present in adipose tissue in conditions of insulin resistance in mice and man. We observed that LCN2 expression and secretion was induced by IFN γ and TNF α in both murine 3T3-L1 adipocytes and human subcutaneous adipocytes. STAT1 and ERKs 1 and 2 signaling pathways mediated the effects induced by IFN γ and the effects of TNF α on LCN2 expression were mediated by both NF- κ B and ERKs 1 and 2 signaling pathways. Our studies of the murine LCN2 promoter identified five STAT1 binding sites and one NF- κ B binding site. Inhibition of ERKs signaling pathway attenuated the stimulatory effects of both IFN γ and TNF α on LCN2 expression. Inhibition of ERKs 1 and 2 reduced the activity of STAT1 and NF- κ B without having any significant effects on the nuclear localization or DNA binding activity of these pro-inflammatory transcription factors. These mechanistic studies provide insight into the modulation of LCN2 that occurs during insulin resistance.

2. RESEARCH DESIGN AND METHODS

2.1. Cell culture

Murine 3T3-L1 preadipocytes were grown to 2 days after confluence in Dulbecco's Modified Eagle's Media (DMEM) with 10% bovine serum. Media was changed every 48 h. 0.5 mmol/l 3-isobutyl-methylxanthine, 1 μ mol/l dexamethasone, and 1.7 μ mol/l insulin (MDI) cocktail were used to induce preadipocytes differentiation in DMEM containing 10% fetal bovine serum (FBS). After 48 h, the media was replaced by DMEM with 10% FBS. For serum deprivation, media was change to DMEM containing 0.3% BSA for 16–20 h before treatment. DMEM was purchased from Sigma. Bovine and FBS were purchased from Hyclone. Human subcutaneous adipocytes in 12-well plates were purchased from Zen-Bio. Cells were serum-deprived for 16 h before treatment. Recombinant mouse and human IFN γ were purchased from R&D Systems. Recombinant mouse and human TNF α were purchased from Gibco. Actinomycin D was purchased from Sigma.

2.2. Preparation of whole cell extracts

Cell monolayers were harvested in a non-denaturing IP buffer that contained 10 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 1% Triton X-100, 0.5% Nonidet P-40, with protease inhibitors 1 μ mol/l phenylmethylsulfonyl fluoride, 1 μ mol/l pepstatin, 50 mU trypsin inhibitory aprotinin, 10 μ mol/l leupeptin, and phosphatase inhibitor 2 mmol/l sodium vanadate. The cells were scraped off the plates and the extract was passed through a 20 g needle three times. The extract was centrifuged at 9500g for 10 min at 4 °C. Supernatants were collected and analyzed with BCA (Pierce) to quantify protein content of whole cell extract.

2.3. RNA analysis

Total RNA was isolated from cell monolayers with RNeasy mini kit (Qiagen). 10 μ l of RNA extract was used for reverse transcription PCR. cDNA was analyzed by delta delta ct real-time PCR with SYBR supermix reagent (Takara) and Applied Biosystem 7900HT system. Cyclophilin A was used as endogenous control. Following primers were used for the real-time PCR: mLipocalin-2 forward TGCAAGTGGCCACACGGAG and reverse GCATTGGTCGGTGGG-GACAGAGA; mCyclophilin A forward CCACTGTCGCTTTTCGCCG and reverse TGCAAACAGCTCGAAGGAGACGC.

2.4. 3T3-L1-adipocyte fractionation

Cell monolayers were harvested in nuclear homogenization buffer containing 20 mmol/l Tris (pH 7.4), 10 mmol/l NaCl, 3 mmol/l MgCl₂, 1 μ mol/l dithiothreitol, with protease inhibitors listed above and 2 mmol/l sodium vanadate. Nonidet P-40 was added to final concentration of 0.15%. Cells were homogenized with 18 strokes in a Dounce homogenizer and centrifuged at 500g for 5 min. Supernatants were transferred to another tube as cytosolic extract. The precipitated nuclear pellets were resuspended in one-half volume of nuclear homogenization buffer and were centrifuged as before. The supernatant was removed and discarded. The majority of the pellet (intact nuclei) was resuspended in nuclear extraction buffer containing 20 mmol/l HEPES (pH 7.9), 150 mmol/l NaCl or 420 mmol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 1 μ mol/l dithiothreitol, 25% glycerol and the with protease inhibitors listed above with 2 mmol/l sodium vanadate. Resuspended nuclei were passed three times through a 20G needle every 10 min and extracted for 30 min on ice. The samples were centrifuged at 9500g at 4 °C for 10 min. The resulting supernatant was a nuclear extract.

2.5. Gel electrophoresis and immunoblotting

Proteins were separated in 7.5% or 10% polyacrylamide gels containing SDS, and transferred to nitrocellulose membrane (Bio-Rad) in 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol. After transfer, the membrane was blocked in 4% milk for 1 h at room temperature, and incubated with primary antibody overnight at 4 °C. Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory) and enhanced chemiluminescence (Pierce). STAT1 antibody was purchased from Cell Signaling Technology. Lipocalin-2 antibody was from R&D System. STAT5A, p65, ERK1/2 antibodies were from Santa Cruz technology. Adiponectin antibody was from Thermo-Pierce. STAT1pY antibody was from Millipore. Active ERK1/2 antibody was from Promega.

2.6. Electrophoretic mobility shift analysis

Double-stranded oligonucleotides were annealed by heating single-stranded oligonucleotides in 95 °C water bath and gradually cooling to room temperature. 4 μ g double-stranded oligonucleotides were labeled with 20 μ Ci [α -P³²]dCTP (PerkinElmer) and 1 μ l of each 5 mM dATP, dTTP, dGTP using Klenow fragment. The mix was incubated for 15 min at 30 °C. The reaction was stopped by adding 1 μ l of 0.5 M EDTA. Labeled double-stranded oligonucleotides were purified with illustra MicroSpin G-25 Columns (GE Healthcare) according to manufacturer's instructions. Radioactivity of labeled oligonucleotides was determined by scintillation counting. Nuclear extracts were incubated with the labeled oligonucleotides for 30 min on ice. The samples were separated in a pre-run (30 min, 200 V at 4 °C) 6% acrylamide/bisacrylamide TBE gel. Nuclear extracts were pre-incubated with 1 μ g antibody (STAT1, STAT3, STAT5A, STAT5B or p65 from Santa Cruz Technology) for 1 h at room temperature for supershift assay. The gels were run at 200 V for ~3.5 h, dried at 80 °C for 45 min with vacuum, and then exposed to Eastman Kodak Co. BioMax MS film with a Kodak BioMax high energy intensifying screen.

2.7. Chromatin immunoprecipitation

ChIP assay was performed with SimpleChIP[®] Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the protocol supplied by company. Cells were serum deprived overnight in DMEM containing 0.3% bovine serum. Cells were treated with IFN γ or TNF α for 30 min in the presence or absence of U0129, an ERK inhibitor (Promega). Adipocytes were cross-linked with formaldehyde and glycine. Chromatin

extracts were prepared after sonication of harvested cells. Immunoprecipitation was performed with specific STAT1 (Cell Signaling Technology) or p65 (Cell Signaling Technology) antibodies. Rabbit normal IgG was used as a negative control. Purified DNA was quantified by real-time PCR with following primers: -266 forward GTGGACAGGCAGTCCAGATCTGAG and reverse AAGATTCTGTCCCTCTCTCCCC; -619 forward CTGTTCTGTAAATGGCAGTGGGG and reverse GGGTGAGCAAGCTGAGAGTGAATG; -676 forward TAAGGACTACGTGGCACAGGAGAG and reverse GAAGTGCCAATACCTTGAGCCCC; -1014 forward GCTTCTGCCAAAGTAAGTGGAGT and reverse TAAGGACTGCAACCTCGGTGTCAT; -1822 forward CTGCCCTGAGTGTGGGTCAAAG and reverse CTGGGAGTGTAGCTCTCTGGTGT; -3171 forward TAGTCCTGCATTAGTTTCAGGC and reverse ACCCAGGTCCAATCCACATGAAGA. 500 nM of each primer and 2 μ l ChIP product were used with SYBR green supermix and ROX buffer (Takara) in 20 μ l PCR reaction. Percentage of input was calculated by the formula: Percentage of input = $2\% \times 2^{(CT - CT_{input})}$.

2.8. Plasmid constructs

A 3470 bp-fragment of lipocalin-2 promoter (-3358 to 112) was cloned from genomic DNA of mouse liver. The fragment was amplified with following primers: forward: GATCGGTACCAAGGGCTCTCCAGTTCTC; reverse: GATCGATATCGGCAGGGATCAAGTTCTGAG. Nucleotides were added to the 5' ends of each primer so that the forward primer could be digested by KpnI and the reverse primer by EcoRV for ligation into the pGL4.27 vector to create pGL4.27-LCN2pro. Plasmids were sequenced for verification of nucleotide sequence.

2.9. Plasmid transfection and luciferase reporter assays

Mature 3T3-L1 adipocytes in six-well plates were transfected with 2 μ g pGL4.27-LCN2pro by using Dharmacon turboFect reagent (ThermoScientific). 2 μ g pRL-TK/renilla vector was co-transfected to control transfection efficiency. After 24 h, the adipocytes were pretreated with U0126 for 30 min and that was followed by IFN γ or TNF α treatment for 24 h. Cell lysis was prepared with passive lysis buffer. Luciferase activity was measured with the Dual Luciferase Reporter System (Promega). Relative luciferase units were calculated by dividing firefly luciferase activity values by renilla luciferase activity. Each condition was performed in triplicate and on three independent batches of adipocytes.

3. RESULTS

3.1. Lipocalin-2 expression and secretion is up-regulated by pro-inflammatory cytokines

Previous studies showed that lipocalin-2 is expressed in adipocytes and increased in obese and diabetic subjects [20]. However, the mechanisms involved in the induction of LCN2 expression are unknown. In our studies, we observed that both IFN γ and TNF α induced the expression and secretion of LCN2. Whole cell extracts and media samples were prepared from 3T3-L1 adipocytes that were untreated or treated for 24, 48, or 72 h. As shown in Figure 1A and B, LCN2 expression and secretion were induced by IFN γ and TNF α treatment within 24 h. After 24 h IFN γ treatment, the cellular levels of LCN2 were induced and remained high. However, the effect of TNF α on LCN2 levels increased over time. Since adiponectin expression and secretion is attenuated by both IFN γ and TNF α [27,28], we used it as control to assess the effectiveness of cytokine treatment. STAT5A was used as a loading control. As expected, cytokine treatment decreased adiponectin levels, but did not affect STAT5A expression. To determine if the observed

effects were dependent on new transcription, we pretreated 3T3-L1 adipocytes with Actinomycin D for 30 min, followed by a IFN γ or TNF α treatment. As shown in Figure 1C, TNF α is a more potent inducer of LCN2 mRNA than IFN γ . In addition, the presence of Actinomycin D inhibited the ability of both inflammatory mediators to induce LCN2 mRNA expression.

3.2. The effects of IFN γ and TNF α on LCN2 expression are additive

Next, we evaluated whether IFN γ and TNF α induced LCN2 expression by activating the same or different signaling pathways in adipocytes. Mature 3T3-L1 adipocytes were treated for 16 h and total RNA was isolated and analyzed by quantitative real-time PCR. As shown in Figure 2A, TNF α is a more potent inducer of LCN2 mRNA than IFN γ . However, the presence of both IFN γ and TNF α always resulted in at least an additive effect on LCN2 expression. This additive effect is also observed with LCN2 protein expression and secretion (Figure 2B). Collectively, these data indicate that IFN γ and TNF α affected LCN2 expression via stimulating different signaling pathways.

3.3. Identification of NF- κ B and STAT1 binding sites in lipocalin-2 promoter

Since the induction of LCN2 expression by IFN γ or TNF α appeared to be mediated by different signaling pathways, we examined several signaling pathways known to mediate the effects of IFN γ and TNF α . We examined the role of STAT1, NF- κ B and ERK1/2 signaling pathways in the ability of IFN γ and TNF α to induce LCN2 expression. First, we performed an analysis to search for potential STAT1 and NF- κ B sites in the promoter of the murine LCN2 gene. Three potential NF- κ B binding sites (Table 1) and nine potential STAT1 binding sites (Table 2) were identified by sequence analysis and experimentally examined by performing both electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP). Only one of the potential NF- κ B binding sites was confirmed to interact with nuclear extracts from TNF α treated adipocytes and binding specificity was assessed by performing cold competition and generating a mutated binding site (Figure 3A and Tables 3 and 4). An increase of cold competitor reduced the interaction between radioactively labeled probe and nuclear protein. A mutated probe (Table 2) did not associate with nuclear protein. A specific p65 antibody was used to perform supershift analysis to determine whether NF- κ B comprised the DNA binding complex. As indicated in Figure 3B, pre-incubation with a p65 antibody resulted in a supershift of the TNF α induced binding, which suggests that the p65 component of NF- κ B is present in the DNA-protein complex. Chromatin immunoprecipitation (ChIP) was also performed to examine the association of p65 with the murine LCN2 promoter. As shown in Figure 3B, p65 bound to the murine LCN2 promoter *in vivo*.

Nine potential STAT1 binding sites were experimentally examined by performing both EMSA and ChIP. EMSA analysis revealed that four of these sites interacted with nuclear protein from IFN γ treated adipocytes (Figure 4A). Specificity was assessed by performing cold competition and generating mutated binding sites (data not shown). Supershift assay was performed with specific STATs antibodies to demonstrate whether these proteins were involved in the DNA binding complex. STAT1 was shown to bind to all four binding sites. To our surprise, STAT5A and STAT5B also interacted with -619, -1822 and -3171 binding sites (Figure 4B). Since growth hormone (GH) is a physiological activator of STAT5A and STAT5B [29], we examined the ability of GH to regulate binding to these STAT sites. However, there was no association detected between labeled oligonucleotides and nuclear protein from GH treated adipocytes (data not shown). When we performed ChIP analysis to test

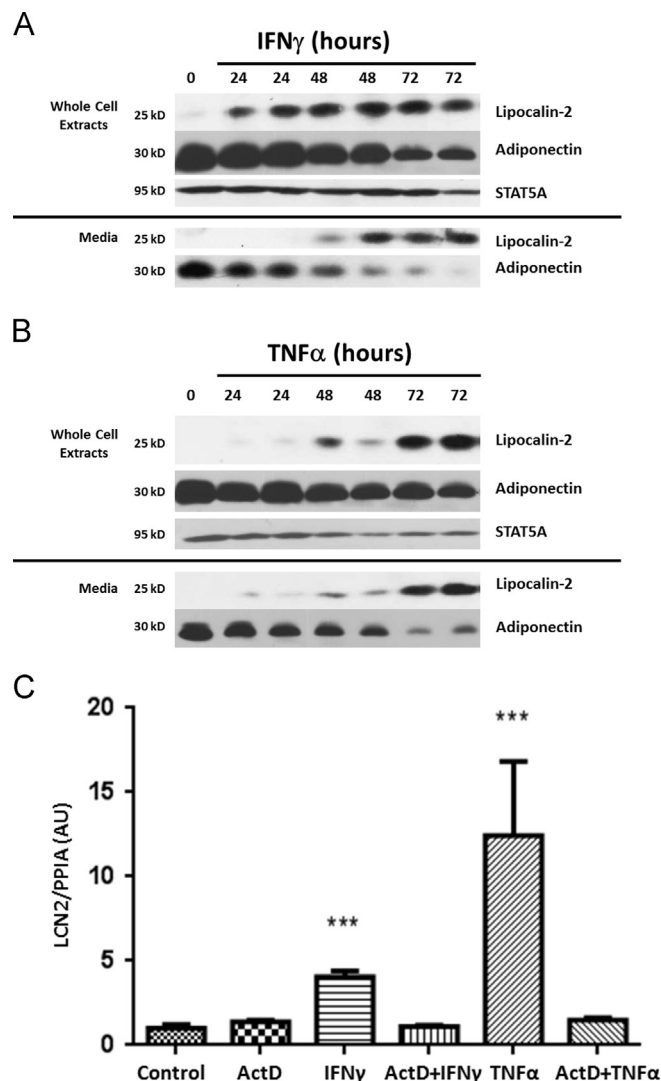


Figure 1: IFN γ and TNF α induce LCN2 expression and secretion in 3T3-L1 adipocytes. A: Mature 3T3-L1 adipocytes were chronically treated with 100 ng/ml IFN γ . Media was collected and whole cell extract was harvested at indicated time. Time 0 indicates no treatment. 250 μ g protein from media samples and 150 μ g of whole cell extracts were subjected to western blot analysis. B: Mature 3T3-L1 adipocytes were chronically treated with 0.5 nM TNF α and samples were analyzed as indicated above. C: Adipocytes were pretreated with 5 μ g/ml Actinomycin D for 30 min, and then treated with IFN γ or TNF α for 16 h. Total RNA was isolated and analyzed with reverse transcription and qPCR. Cyclophilin A was used as an endogenous control. These are representative experiments independently performed three times.

these STAT binding sites, we confirmed the four binding sites that were validated by EMSA and observed STAT1 binding at one additional site (Table 2). Of note, this binding site at -1014 was not positive with nuclear protein in vitro, but was verified by ChIP using a STAT1 antibody (Figure 4C). For all the five STAT1 binding sites, we did not observe any IFN γ induced interaction with STAT5 in vivo (data not shown).

3.4. ERK signaling modulates the ability of TNF α and IFN γ to induce LCN2 expression without having significant effects on STAT1 and NF- κ B nuclear translocation or DNA binding activity

Besides activating transcription factors like STAT1 and NF- κ B, both IFN γ and TNF α acutely activate ERKs 1 and 2 (Figure 5A). Mature adipocytes were pretreated with U0126, a specific ERK inhibitor for 30 min and then nuclear translocation and tyrosine phosphorylation of STAT1 was examined after an IFN γ or TNF α treatment for 20 min. As shown in Figure 5A, the IFN γ induced nuclear translocation and tyrosine phosphorylation of STAT1 was unaffected by ERK inhibition. TNF α induced the nuclear translocation of p65 that was also unaffected by

ERK inhibition. The efficacy of ERK inhibition is shown by using a phospho-specific antibody to assess ERK activation. Also, note that IFN γ induced a modest increase in the nuclear content of STAT5A, but did not affect the content of the nuclear protein DBC1. Although, ERK inhibition did not affect the ability of these pro-inflammatory cytokine to induce nuclear translocation of STAT1 and p65, it did have substantial effects on the ability of these cytokines to induce LCN2 expression. As shown in Figure 5B, the induction of LCN2 mRNA levels by IFN γ and TNF α was repressed by ERK inhibition. However, EMSA analysis revealed no alterations in binding to the NF- κ B site in the LCN2 promoter in vitro and ChIP analysis suggested that NF- κ B binding to this site was not significantly alerted in vivo ($P=0.0538$). We also examined the STAT1 binding to the LCN2 promoter under conditions where ERK was inhibited. As shown in Figure 5D, there was no difference in binding in vitro or in vivo for any of the STAT1 binding sites identified. To determine if ERK inhibition modulated LCN2 promoter activity in adipocytes, we cloned ~3.5 kb of the murine LCN2 promoter into a pGL4.27 vector expressing firefly luciferase. This vector was transiently

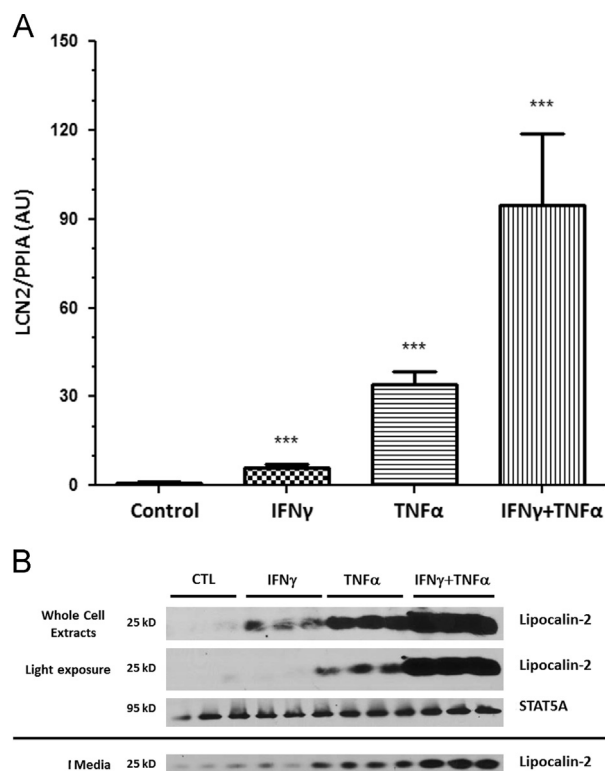


Figure 2: The effect of IFN γ and TNF α on LCN2 expression are additive. Mature 3T3-L1 adipocytes were treated with 100 ng/ml IFN γ or 0.5 nM TNF α or both cytokines. A: RNA was isolated 16 h later and subject to reverse transcription and qPCR. B: Whole cell extracts and media samples were collected after a 24 h treatment. 250 μ g of protein from media samples and 150 μ g of whole cell extracts were analyzed by western blot analysis. These are representative experiments performed with three biological replicates and independently performed on three batches of cells.

Gene	Position	Sequence	TNF α responsive	
			EMSA	ChIP
Consensus		GGG NNN NNC C		
Lipocalin-2	–266 to –247	GC CCT GGG AAT GTC CCT CTG	Yes	Yes
Lipocalin-2	–713 to –694	GG AGA GGG TGA GTC CCT GAG	No	No
Lipocalin-2	–1795 to –1774	GAA CTT GGG GTC TCC CAT GTG C	No	No

Table 1: NF- κ B binding sites in lipocalin-2 promoter.

transfected into 3T3-L1 adipocytes. A Renilla-TK vector was co-transfected to control for transfection efficiency. One day after transfection, the cells were treated with IFN γ or TNF α for 24 h in the presence or absence of the ERK inhibitor. A dual-luciferase reporter assay was performed to quantitate the expression of luciferase. The results clearly showed that the ~3.5 kb segment, immediately upstream of the transcription start site within the LCN2 promoter was sensitive to both pro-inflammatory cytokines. The promoter activity was highly similar to what we observed for the induction of LCN2 mRNA (refer to Figures 2 and 5). However, U0126 pretreatment abrogated the stimulation of luciferase expression by both IFN γ and TNF α indicating that the inhibition of ERKs impaired the transcription activation activity of STAT1 and NF- κ B (Figure 6).

Gene	Position	Sequence	IFN γ responsive	
			EMSA	ChIP
Consensus		TTC N ₂₋₄ GAA		
Lipocalin-2	–160 to –142	G TAT TTC AAC AGA ATG TAC	No	No
Lipocalin-2	–619 to –602	GA CAC TTC CAG GAT AAT C	Yes	Yes
Lipocalin-2	–676 to –697	GT CTG TTC CTG TAA ATG GCA	Yes	Yes
Lipocalin-2	–705 to –688	GT GAG TCC CTG AGA GTT C	No	No
Lipocalin-2	–1014 to –994	CCC ACT TTC CCC AAG GGC TCC	No	Yes
Lipocalin-2	–1822 to –1806	GGT TGT TTC TTT GTA CT	Yes	Yes
Lipocalin-2	–3132 to –3115	GGA TCC TTG AGA TGC AAC	No	No
Lipocalin-2	–3159 to –3143	AG AGT TTC TGG ATC CGA	No	No
Lipocalin-2	–3171 to –3152	CC AGT TTT TGG AAG AGT TTC	Yes	Yes

Table 2: STAT1 binding sites in lipocalin-2 promoter.

3.5. IFN γ and TNF α induce lipocalin-2 expression in human subcutaneous adipocytes

An in silico analysis of the human LCN2 promoter revealed five potential STAT1 binding sites at –856, –885, –1405, –1934, and –3610. As shown in Table 5, sequences of all these potential binding sites are largely conserved between mice and humans. Furthermore, the distribution of these sites in the human promoter is comparable with the STAT1 binding sites we characterized in the murine LCN2 promoter. We also identified one potential NF- κ B binding site in the human LCN2 promoter at –176 and this is completely conserved between humans and mice (Table 5). Our in silico analysis of the human LCN2 promoter strongly suggests that STAT1 and NF- κ B also mediate the induction of LCN2 expression in human adipocytes. In order to determine if the regulation we observed in murine 3T3-L1 adipocytes also occurred in human cells, we purchased human adipocytes. Human IFN γ and TNF α were used to treat human subcutaneous adipocytes for 24 h. As shown in Figure 7A and B, the expression of LCN2 was up-regulated by both IFN γ and TNF α in human adipocytes.

4. DISCUSSION

The endocrine properties of adipocytes are one of its most critical functions [2]. Leptin and adiponectin are two hormones that are produced exclusively from adipocytes in mouse and man. Like LCN2, these hormones are also implicated in modulating insulin sensitivity and whole body energy balance [2]. Although it is known that LCN2 expression can be highly induced and secreted from adipocytes and LCN2 is significantly regulated in condition of mouse and human obesity, the mechanisms involved in this induction are poorly understood. Lipocalin-2 protein level are elevated in ob/ob mice and db/db mice [20]. In agreement with these animal experiments, human studies revealed that circulating LCN2 concentrations positively correlate with adiposity, triglyceride concentration, blood glucose level and insulin resistance [21,22]. Yet, LCN2 is negatively related to high-density lipoprotein cholesterol [22]. However, there is very little information on the mechanisms involved in the modulation of LCN2 expression. It has been reported that Interleukin-1 beta (IL-1 β) induces LCN2 expression

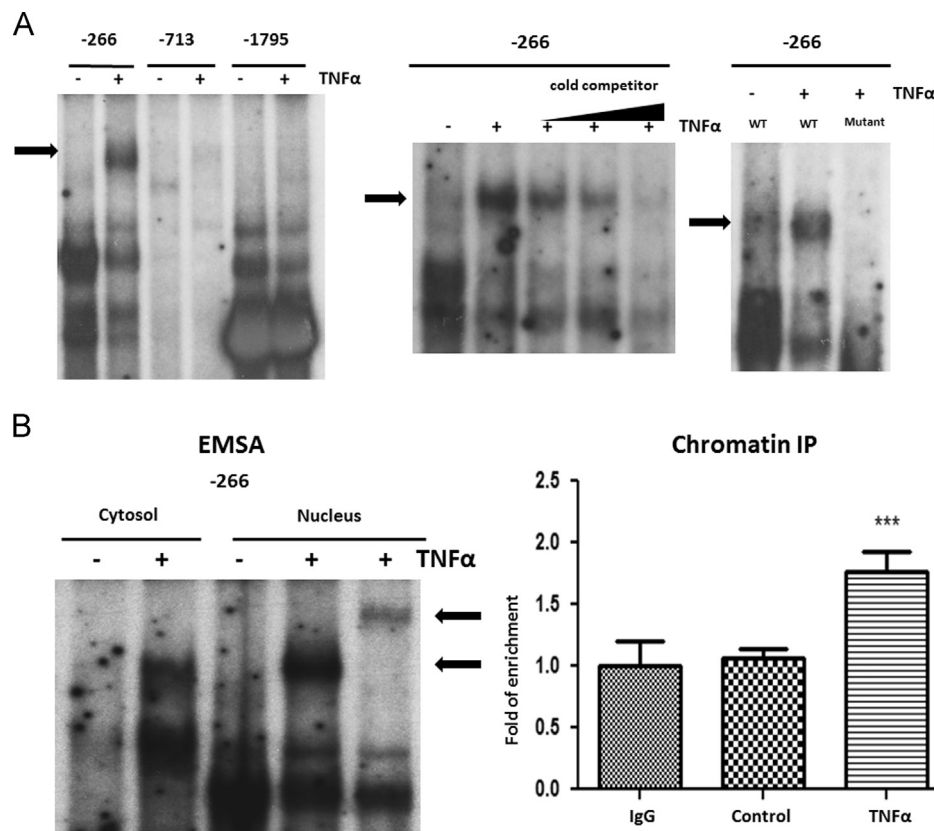


Figure 3: TNF α induces NF- κ B binding to LCN2 promoter. A: Mature 3T3-L1 adipocytes were treated with 0.5 nM TNF α for 20 min. Cytosolic and nuclear extracts were separated from treated or untreated mature adipocytes. 10 μ g of each extract was incubated with 50,000 cpm/ μ l 32 P-labeled double-stranded oligonucleotides of murine LCN2 promoter. Protein-DNA complexes were resolved by EMSA. The TNF α -responsive shift is indicated with an arrow (left panel). For cold competition, nuclear extracts were incubated with 50,000 cpm/ μ l 32 P-labeled probe and 2 μ l unlabeled oligonucleotides of different concentrations (0.4 μ M, 2 μ M, 10 μ M) (middle panel). Nuclear extracts were also incubated with 50,000 cpm/ μ l 32 P-labeled double-stranded probe of LCN2 promoter or probe of mutated sequence (right panel). B (left panel): Cytosolic and nuclear extracts were prepared from mature adipocytes treated with TNF α for 20 min. For supershift assay, the nuclear protein was pre-incubated with 1 μ g indicated antibody for 1 h, and then incubated with 32 P-labeled probe and protein-DNA complexes were resolved by EMSA. B (right panel): 3T3-L1 adipocytes were treated or untreated with 0.5 nM TNF α for 30 min, and then cross-linked with formaldehyde. Chromatin extracts were prepared and subject to immunoprecipitation with IgG (Negative control) or p65 antibody. IP products were analyzed by qPCR. Percentage of input was calculated and normalized to negative control. $P < 0.01$. These are representative experiments independently performed three times.

Gene	Position	Mutated sequence	IFN γ responsive (EMSA)
Lipocalin-2	-619 to -602	GA CAC CTC CAG GCT AAT C	No
Lipocalin-2	-676 to -657	GT CTG TAC CTG TCA ATG GCA	No
Lipocalin-2	-1822 to -1806	GGT TGT CTC TTT GTC CT	No
Lipocalin-2	-3171 to -3152	CC AGT CTC TGG ACG AGT TTC	No

Table 3: Mutated STAT1 binding sites.

Gene	Position	Mutated sequence	TNF α responsive (EMSA)
Lipocalin-2	-266 to -247	GC CCT GAG AAT GTA CCT CTG	No

Table 4: Mutated NF- κ B binding site.

through NF- κ B and JAK2 mediated signaling pathways [30]. However, these observations are solely based on the use of inhibitors and no binding sites or promoter activity was examined. Other studies indicate that expression of LCN2 is up-regulated by agents that are associated with insulin resistance, including lipopolysaccharide (LPS) [31], glucocorticoid [32], and insulin [33]. Insulin sensitizers, such as thiazolidinedione, down-regulate LCN2 expression [23]. Our studies provide the first mechanistic data on the identification and binding of two

transcription factors (STAT1 and NF- κ B subunit p65) to the binding sites of the LCN2 promoter in vitro and in vivo. These results also reveal a role of serine/threonine kinases ERKs 1 and 2 on the induction of LCN2 by pro-inflammatory cytokines that can be produced from immune cells in adipose tissue to inhibit insulin signaling and induce insulin resistance in adipocytes.

Our studies demonstrate that IFN γ and TNF α induce LCN2 expression in both murine and human adipocytes. We provide evidence that STAT1 plays a role in the effects of IFN γ , while TNF α induces LCN2 expression via NF- κ B signaling pathways. Sequence analysis of the LCN2 promoter identified nine potential STAT binding sites and three potential NF- κ B binding sites. Binding of these transcription factors was assessed by both EMSA and ChIP analysis. These studies revealed the binding activity of a NF- κ B site and five STAT1 binding sites in the LCN2 promoter. Transfection experiments with a promoter construct confirmed that the region containing these binding sites in LCN2 promoter mediated the increase of gene expression after IFN γ and TNF α treatment. Moreover, inhibition of ERKs 1 and 2 activation attenuated the stimulatory effects of both cytokines. Our data suggest that ERK signaling is required for the maximal transactivation activity of STAT1 and NF- κ B, but ERK inhibition did not have significant effect on nuclear localization or DNA binding activity of these transcription factors. We hypothesize that ERK has direct effects on the serine phosphorylation of

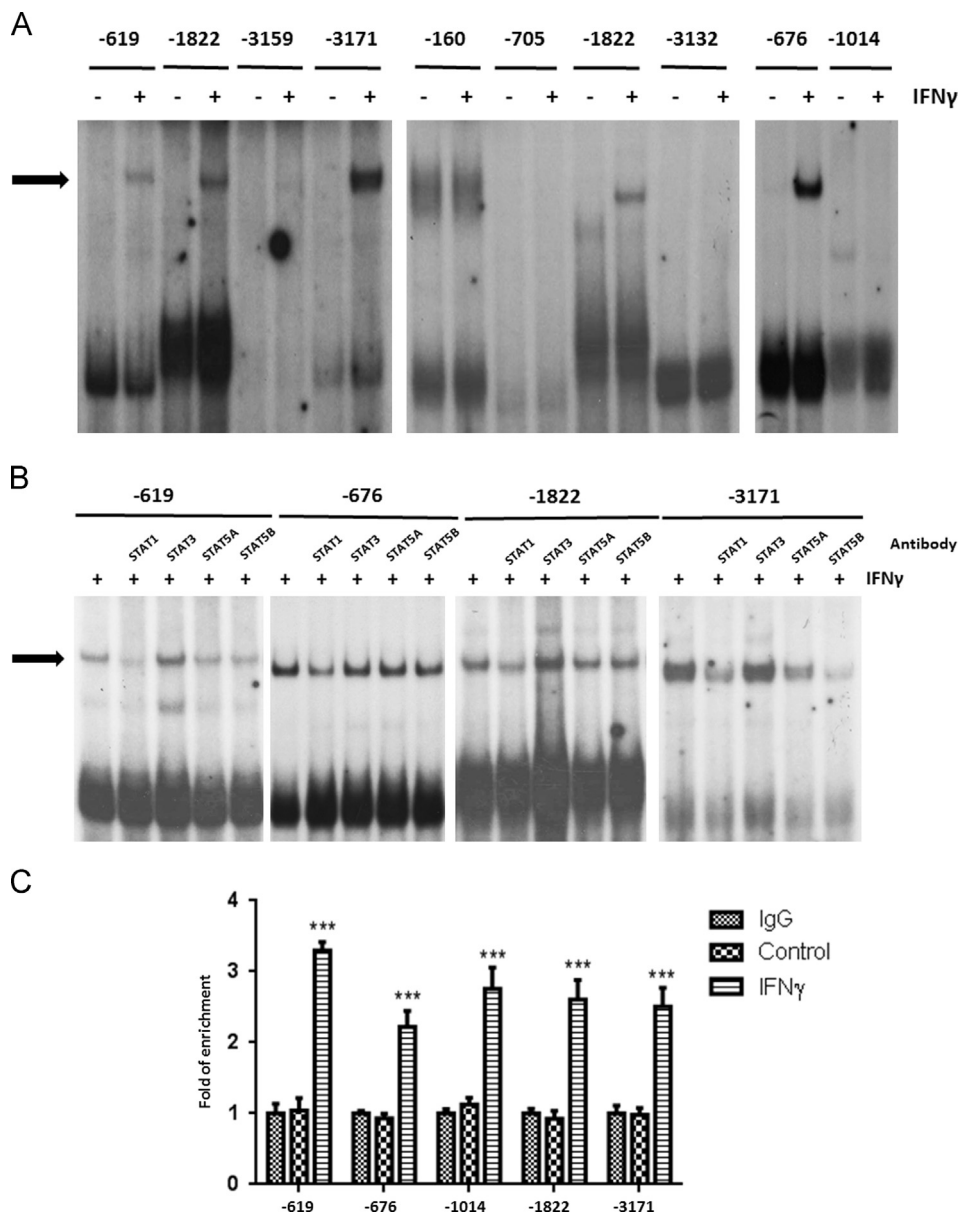


Figure 4: IFN γ induces STAT1 binding to LCN2 promoter. A: Mature 3T3-L1 adipocytes were treated with 100 ng/ml IFN γ for 20 min for the preparation of cytosolic and nuclear extracts. There was no binding in any cytosolic extracts (data not shown). 10 μ g of nuclear extract was incubated with 50,000cpm/ μ l 32 P-labeled double-stranded oligonucleotides of murine LCN2 promoter. The protein–DNA complexes were resolved by EMSA. The IFN γ responsive shift is indicated with an arrow. B: Nuclear extracts were prepared from mature adipocytes treated with IFN γ for 20 min. For supershift assay, the nuclear protein was pre-incubated with 1 μ g indicated antibody for 1 h, and then incubated with 32 P-labeled probe. Protein–DNA complexes were resolved by EMSA. C: 3T3-L1 adipocytes were treated or untreated with 100 ng/ml IFN γ for 30 min, and then cross-linked with formaldehyde. Chromatin extracts were prepared and subject to immunoprecipitation with IgG (Negative control) or the STAT1 antibodies indicated in the figure. IP products were analyzed by qPCR. Percentage of input was calculated and normalized to negative control. *** P < 0.01. Each panel is representative experiment that was independently performed three times.

p65 or STAT1 and future studies will address this question. Previous studies have demonstrated that serine phosphorylation of STAT1 is required for its maximum transactivation activity [34]. Also, several serine phosphorylation sites have been identified in NF- κ B subunit p65 [35–39]. Although our preliminary studies suggest these transcription factors are serine phosphorylated by ERKs, we have not examined whether this phosphorylation mediates the ERKs dependent effects on the LCN2 expression. Collectively, our findings suggest that ERKs signaling pathways crosstalk with the STAT1 and NF- κ B signaling pathways in adipocytes.

One drawback related to our studies is that the functions of LCN2 still need to be determined. Although several groups have studied the LCN2

null mice, the results of these studies are inconsistent. Previous research have shown important roles of LCN2 in the transportation of hydrophobic molecules, in limiting of bacterial growth [18] and modulating degradation of matrix turnover enzyme MMP-9 [15]. The versatile functions of LCN2 make it difficult to assess the primary reason that accounts for the different phenotypes of the LCN2 null mice. Some data shows induction of PPAR γ and adiponectin by LCN2 [23] suggesting that adipokine may be beneficial. Yet, other studies indicate improved insulin action in LCN2 knockout cells [20], indicating that LCN2 is associated with metabolic dysfunction. Although it is clear that LCN2 affects adipocyte properties and participates in the regulation of insulin sensitivity, opposite effects of LCN2 have been reported.

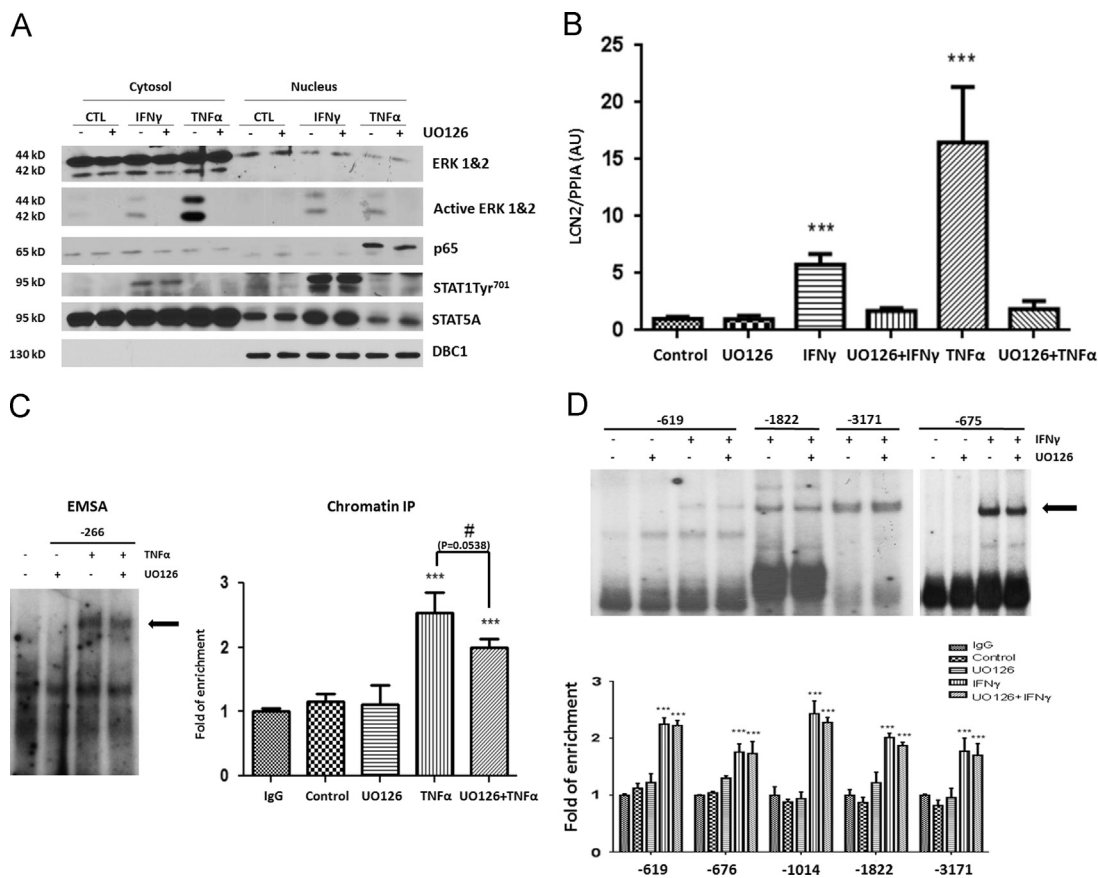


Figure 5: ERK inhibition attenuates the ability of IFN γ and TNF α to induce LCN2 without modulating the nuclear translocation or DNA binding activity of STAT1 and NF- κ B. A: Cytosolic and nuclear extracts were prepared from mature 3T3-L1 adipocytes treated or untreated with IFN γ or TNF α for 20 min in the presence or absence of 50 μ M UO126. 100 μ g protein of each sample was subject to Western blot analysis. B: Mature 3T3-L1 adipocytes were pretreated with 50 μ M UO126 for 30 min, and then treated with IFN γ or TNF α for 16 h. Total RNA was isolated and analyzed with reverse transcription and qPCR. Cyclophilin A was used as endogenous control. $P < 0.01$. C: (left panel): Nuclear extracts were separated from adipocytes untreated or treated with TNF α for 20 min in the presence or absence of UO126. 10 μ g of nuclear extract was incubated with 50,000 cpm/ μ l 32 P-labeled double-stranded oligonucleotides of murine LCN2 promoter. Protein–DNA complexes were resolved by EMSA. C (right panel): 3T3-L1 adipocytes were untreated or exposed to TNF α for 30 min in the presence or absence of UO126, and then cross-linked with formaldehyde. Chromatin extracts were prepared and subject to immunoprecipitation with IgG (Negative control) or p65 antibody. IP products were analyzed by qPCR. Percentage of input was calculated and normalized to negative control. $P < 0.01$. D: (upper panel): Nuclear extracts were separated from untreated adipocytes or treated with IFN γ for 20 min in the presence or absence of UO126. Protein–DNA complexes were resolved by EMSA as described above. (Lower panel): 3T3-L1 adipocytes were untreated or exposed to IFN γ for 30 min (–/+ UO126), and then cross-linked with formaldehyde. Chromatin IP experiments were performed with STAT1 antibody for each of the LCN2 promoter sites indicated. $P < 0.01$. Each panel of this figure is a representative experiment that was performed three times in independent batches of cells.

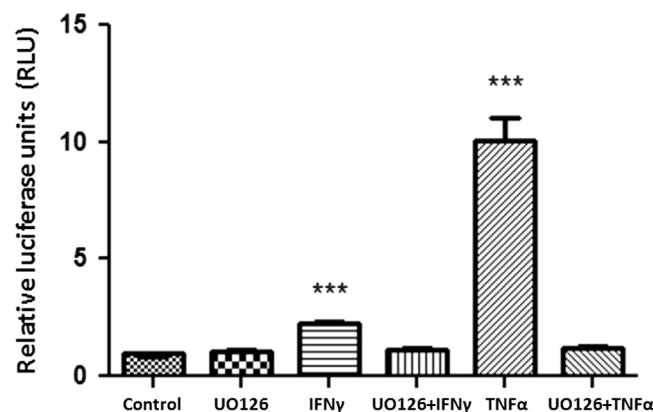


Figure 6: ERKs inhibition impairs the induction of LCN2 promoter by both IFN γ and TNF α in adipocytes. Mature 3T3-L1 adipocytes were transiently transfected with murine lipocalin-2 promoter (-3358 to 112)/luciferase plasmid. A TK/renilla vector was used as control for transfection efficiency. After 24 h post-transfection, adipocytes were pretreated with 50 μ M UO126 for 30 min and then stimulated with IFN γ or TNF α for 24 h. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity. $P < 0.01$. Each condition was performed in triplicate. These are representative experiments independently performed on three different groups of adipocytes.

	Murine		Human	
	(ENSMUST0000050785)		(ENST00000277480)	
STAT1 (TTCN ₂ -GAA)	— 619	TTC ^{CA} GGA ^{TA} AAT	— 856	TTC ^{AAT} GGA ^{AA} AA
	— 676	TTCC ^T GA ^{AAA}	— 885	TTCC ^A GAA ^{ACC}
	— 1014	TTCA ^{ATT} GAT	— 1405	TTCTG ^{GA} GA
	— 1822	TTCTTT ^{GTA} C	— 1934	TTCCG ^{GA} GTA
	— 3171	TTCTGGA ^{AGA}	— 3610	TTCTG ^{GA} AGATG
NF-κB (GGGN ^{NNNN} NCC)	— 266	GGGAATG ^{TCCC}	— 176	GGGAATG ^{TCCC}

Table 5: A comparison of STAT1 and NF-κB binding sites in the murine and human lipocalin-2 promoters.

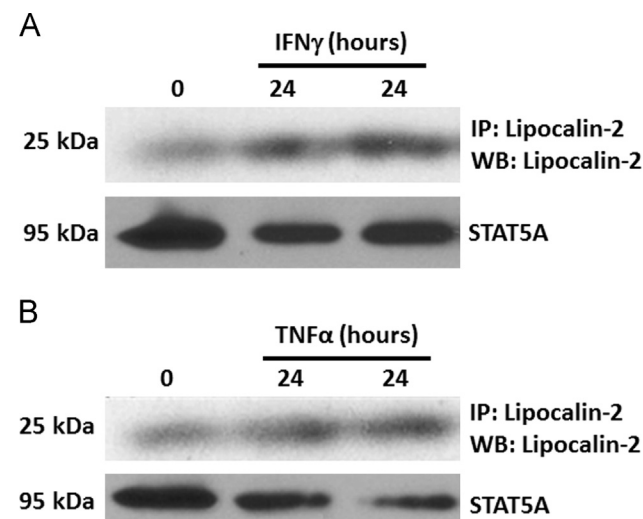


Figure 7: IFN γ and TNF α induce LCN2 expression in human adipocytes. Human subcutaneous adipocytes, purchased from Zen-Bio, were treated with 100 ng/ml IFN γ or 0.5 nM TNF α . Whole cell extracts were harvested after 24 h. Time 0 indicates no treatment. 300 μ g protein was used to perform immunoprecipitation (IP) with a human lipocalin-2 antibody. IP products were analyzed by western blot with the same antibody. This experiment was repeated twice on two different batches of cells.

Therefore, our studies on the regulation of LCN2 expression have merit and allow us to speculate the inflammatory cytokines we have shown to induce LCN2 may play a role in metabolic dysfunction.

In summary, LCN2 expression and secretion is induced by IFN γ and TNF α . Both of these pro-inflammatory cytokines are likely pathological mediators of increased LCN2 expression that occurs in adipose tissue in conditions of insulin resistance in mouse and man. Our mechanistic studies reveal that STAT1, NF-κB and ERKs signaling pathways participate in the modulation of LCN2 expression in vitro and in vivo. Future studies will be needed to determine if these transcriptional factors are essential for the induction of LCN2.

CONFLICT OF INTEREST

Neither author has any conflicts of interest.

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PZ and JS designed and conducted the experiments. PZ wrote the manuscript and JS edited to produce the final version. We would like to thank Anik Boudreau for cell culture support and the rest of the Stephens lab for feedback at lab meetings.

Dr. Jackie Stephens is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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